

# Identification and Characterization of *Bacillus anthracis* Spores by Flow Cytometry

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## Abstract

Rapid and accurate detection of *Bacillus anthracis*, the causative agent of anthrax, remains an active area of research due to the continued threat of bioterrorist attack. The ability to differentiate *Bacillus anthracis* spores from spores belonging to other *Bacillus* species is important for the development of spore-based detection methods. Furthermore, not all *Bacillus anthracis* strains are fully virulent and the ability to rapidly determine the potential virulence of the spore is also important. Thus far, no spore-based method exists that can simultaneously satisfy both criteria. We conjugated a previously identified synthetic peptide to the fluorescent protein R-phycoerythrin to make a reagent that differentiates among *Bacillus* species. As expected, the conjugate selectively labeled *Bacillus anthracis* spores but could not distinguish between spores from fully virulent or minimally virulent strains. In response, our laboratory developed a fluorescent antibody-based assay that can be used to detect protective antigen protein associated with the surfaces of *Bacillus anthracis* spores. The two methods were combined in a two-color flow cytometric assay capable of simultaneously identifying the spore bacterial species as well as the relative virulence of the spore. This assay is novel in that *Bacillus anthracis* spores from protective antigen-producing strains can now be distinguished from protective antigen-negative strains. Surface protective antigen was detected in *Bacillus anthracis* spores that were prepared as long as four years ago; however, prolonged storage of spores was

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found by transmission electron microscopy to cause degradation of the exosporium and a loss of binding to the species-specific peptide. Based on the results of this study, we classified a set of prototypical flow cytometry dot-plot patterns that can be used to predict the species and relative virulence of unidentified samples of *Bacillus* spores in as little as one hour.

## Introduction

*B. anthracis* is a Gram positive, rod-shaped bacterium which forms spores that are resistant to environmental changes such as moderate heating and radiation (Nicholson et al. 2000). *B. anthracis*, *B. cereus*, *B. thuringiensis*, and *B. mycoides* collectively form the *B. cereus* group of bacteria, which exist ubiquitously in nature and are genetically related. One of the only distinguishing features among members of the *B. cereus* group is plasmids that encode for virulence factors (Helgason et al. 2000). The *B. anthracis* pXO1 plasmid encodes for protective antigen (PA), edema factor (EF), and lethal factor (LF); three proteins which interact synergistically to form edema toxin (PA and EF) and lethal toxin (PA and LF) (Abrami et al. 2005). Fully virulent isolates of *B. anthracis* also harbor the pXO2 plasmid that encodes for an anti-phagocytic poly-D-glutamic acid capsule (Green et al. 1985). The *B. anthracis* Ames strain possesses both virulence plasmids, whereas minimally virulent Sterne and Pasteur strains lack pXO2 or pXO1, respectively.

Depending on the method of contact with *B. anthracis* spores, infection can result in cutaneous, gastrointestinal, or pulmonary anthrax. The severity of disease is dependent on the spore's ability to evade host immune responses, resume vegetative growth, and secrete edema and lethal toxin. Pulmonary anthrax is the form most commonly associated with bioterrorism because spores can be aerosolized and their size is optimal for deposition in the lungs. Once

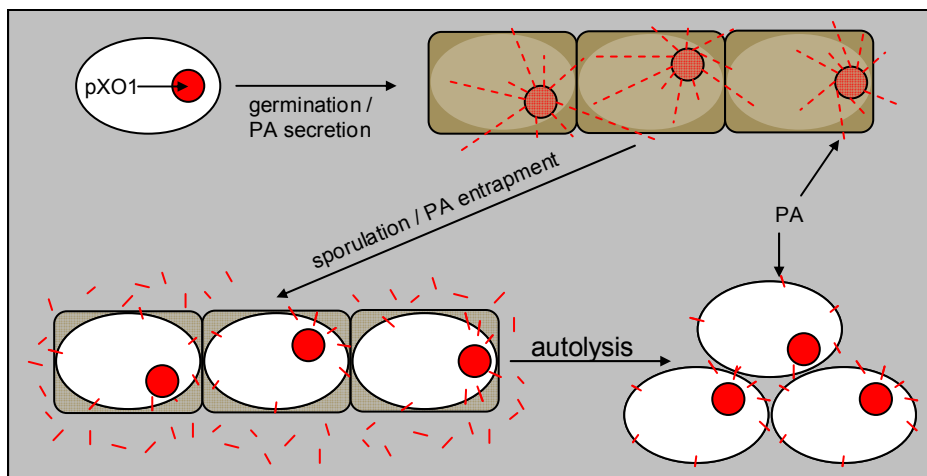
inhaled, the *B. anthracis* spores are transported by alveolar macrophages to lymph nodes, where germination occurs. The bacteria then replicate to very high numbers in the blood and secrete the toxin which ultimately leads to death of the host. Inhaling as few as 10,000-20,000 *B. anthracis* spores is considered a lethal dose. Since the human immune system cannot effectively combat the systemic infection caused by this pathogen, external detection systems that are sensitive to low spore concentrations are critical to our national security.

While the polymerase chain reaction (PCR) (Patra et al. 2002) and traditional culture (Leise et al. 1959) are still heavily relied upon for accurate identification of *B. anthracis*, these methods typically suffer from extensive sample preparation and high cost. Spore-based detection strategies have grown in popularity because surface antigens can be rapidly detected on intact spores using relatively simple labeling procedures (Edwards et al. 2006). However, many of these systems are not selective against other members of the *B. cereus* group (Kamboj et al. 2006; Stopa 2000). Through a phage display screening process, short peptide fragments that exhibited species-specific binding to *Bacillus* spores were discovered (Turnbough 2003; Williams et al. 2003), and numerous reports showed them to be sensitive and selective (Acharya et al. 2007; Pai et al. 2005). Peptides are attractive candidates for pathogen detection because of their structural simplicity and durability, as well as their desirable binding properties as selected for by the phage display process. Furthermore, such ligands can be synthesized to include reactive moieties for straightforward attachment onto sensing platforms.



One such peptide, ATYPLPIRGGGC (abbreviated ATYP), was conjugated to R-phycoerythrin (RPE) through its terminal cysteine residue and found by flow cytometry (FCM) to have nanomolar sensitivity for *B. anthracis* spores and minimal cross-reactivity with closely related species, *B. cereus* and *B. thuringiensis* (Fig. 1). However, like other rapid-response detectors, ATYP-RPE could not distinguish among spores from different strains of *B. anthracis* (Williams et al. 2003).

Our search for a *B. anthracis* spore-associated antigen capable of differentiating strains led us to the PA protein expressed by strains harboring the pXO1 plasmid (Cote et al. 2005; Welkos et al. 2001). Presumably PA is secreted during the germination process and non-covalently entrapped in the spore coat(s) and exosporium after sporulation (Fig. 2). Since the pXO1 plasmid encodes for several virulence factors, surface PA can be considered a virulence marker for *B. anthracis* spores.



**Figure 2.** Upon recognition of a nutrient-rich environment, the *B. anthracis* spore resumes germination and subsequently secretes toxin encoded by the pXO1 plasmid. As the surrounding nutrients expire, the newly replicated pre-spores mature in a process known as sporulation, at which point some of the secreted PA gets entrapped in the outer spore layers. As a final step, autolysis of the “mother” cell releases the spores to the surrounding medium.

Here, we report on a two-color flow cytometric assay which couples a FITC-conjugated antibody-based PA detection scheme to the *B. anthracis* species-specific conjugate, ATYP-RPE. To our knowledge this is the first instance of spore surface PA detection by FCM. And while others have used conventional FCM to characterize *Bacillus* spores (Kamboj et al. 2006; Stopa 2000), we present here the first such two-color method. We demonstrate the potential of this technology by successfully differentiating spores from PA-producing (*B. anthracis* Ames and Sterne) and PA-negative (*B. cereus* and *B. thuringiensis*) strains.

## Materials and Methods

**Bacterial stocks.** *Bacillus anthracis* Sterne (Colorado Serum Company, Denver, CO), *B. anthracis* Ames, *B. cereus*, and *B. thuringiensis* (BMI, Columbus, OH) were grown, sporulated, and purified according to previously established methods (Ireland and Hanna 2002; Kim and Goepfert 1974; Tamir and Gilvarg 1966). Purified spore lots were plated using the serial dilution method to determine colony forming units per milliliter (CFU/ml).

**ATYP-RPE assay.** A peptide with sequence ATYPLPIRGGGC was chemically synthesized and purified by high-performance liquid chromatography (GenScript Corporation), then conjugated to RPE (Invitrogen) through the heterobifunctional cross-linker sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (S-SMCC, Pierce) (Hermanson 1995). Spores ( $10^6$  CFU/ml) were first rinsed with calcium / magnesium-free PBS buffer, 1% fetal bovine serum, pH 7.2 (staining buffer), then mixed with 25  $\mu$ l of 2  $\mu$ M ATYP-RPE and incubated for one hour at 37°C. Unbound conjugate was removed by repeated wash / centrifuge steps using 1X PBS, pH 7.4 (wash buffer). The ATYP-RPE labeled spores were fixed in 2% paraformaldehyde (PFA) prior to analysis.

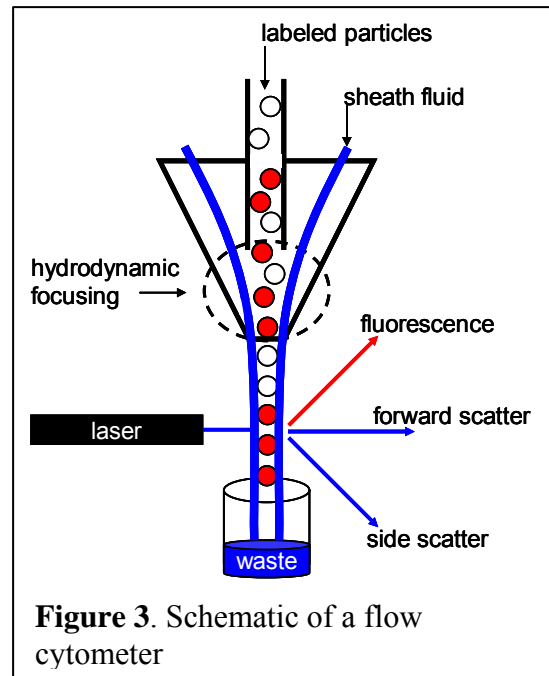
**Surface PA assay.** 2  $\mu$ l of the mouse monoclonal to *B. anthracis* PA (BAP0101, 2.00 mg/ml, abcam) was mixed with spores ( $10^6$  CFU/ml) in 23  $\mu$ l of staining buffer and incubated for one hour at 37°C. Excess anti-PA mAb was removed by repeated wash / centrifuge steps using wash buffer. Bound anti-PA was detected by incubating with 25  $\mu$ l of a 1:100 dilution of fluorescein isothiocyanate (FITC)-conjugated AffiniPure goat anti-mouse IgG (H+L) (Jackson ImmunoResearch) for one hour at 37°C. Again, excess anti-IgG-FITC was removed by wash / centrifuge steps using wash buffer. The anti-IgG-FITC / anti-PA labeled spores were fixed in 2% PFA prior to analysis. As a negative control for surface PA, unlabeled spores were incubated with anti-IgG-FITC (no anti-PA treatment) and processed similarly.

**Two-color assays.** Anti-PA mAb treated spores were prepared according to the procedure established in the previous section. Detection reagents (2  $\mu$ M ATYP-RPE and 1:100 anti-IgG-FITC) were then applied in the following manner: spores ( $10^6$  CFU/ml) were incubated with 25  $\mu$ l anti-IgG-FITC followed by incubation with 25  $\mu$ l ATYP-RPE. Both incubation steps were carried out for one hour at 37°C, and excess detection reagents were removed by wash / centrifuge steps performed before and after incubation with ATYP-RPE. Doubly-labeled spores were fixed in 2% PFA and analyzed by FCM (FACSCalibur instrument and CellQuest Pro software, Becton Dickinson Biosciences) and confocal microscopy (Leica TCS SP2 AOBS confocal laser scanning microscope).

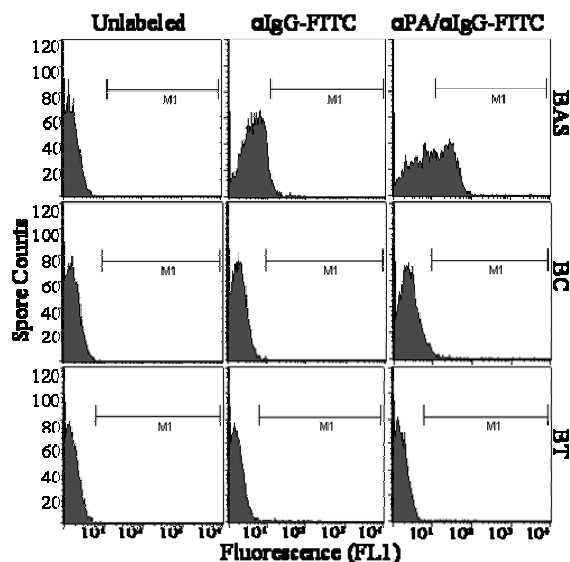
## **Results & Discussion**

**Detection of spore-associated PA by FCM.** Flow cytometry is a high-throughput technique for counting, examining, and sometimes sorting fluorescently-labeled particles contained in a stream of fluid (Fig. 3). A single wavelength laser beam is directed through a hydrodynamically

focused stream of fluid. Several detectors positioned along or perpendicular to the laser beam pick up light scattered off the particles and correlate it to size (forward scatter), inner complexity (side scatter), or fluorescence. In this way FCM allows for simultaneous multi-parameter analysis of physical and chemical characteristics of single particles as they pass through the beam path. If multiple fluorescent labels are used, additional chemical information can be extracted from a single



sample. When viewing a flow cytometry histogram, fluorescence is usually scaled logarithmically on the x-axis and relative abundance is found on the y-axis. As shown in Figure 4, positive fluorescence is validated here from a series of controls including unlabeled spore controls, negative controls (- anti-PA / + anti-IgG-FITC), and species controls (*B. cereus*, BC and *B. thuringiensis*, BT).



**Figure 4.** Representative FCM data set for the surface PA assay. Anti-PA mAb was selective for *B. anthracis* Sterne spores (BAS, 42% positive) against *B. cereus* (BC, 1.3% positive) and *B. thuringiensis* spores (BT, 0.24% positive). M1 = FL1 cutoff marker, which defines a positive event.

For all species, unlabeled spores possessed a minimal level of intrinsic fluorescence, and only *B. anthracis* Sterne (BAS) showed any nonspecific fluorescence associated with binding of anti-IgG-FITC. After establishing a cutoff value for positive fluorescence based on these controls, PA was detected on the surface of BAS spores. As expected, PA was not detected on the surface of BC or BT spores since neither species harbor the pXO1 plasmid.

Recent work supporting the presence of PA on the surface of pXO1-producing strains of *B. anthracis* includes both indirect and direct detection methods. Indirect measurement of surface PA on Ames spores was accomplished by monitoring phagocytosis (rate was enhanced) and germination (rate was inhibited) after treatment by an anti-PA Ab (Welkos et al. 2002). Surface PA was directly measured throughout the spore structure using immunogold labeling / electron microscopy (immunoEM) on thin sections (Cote et al. 2005; Welkos et al. 2001). After getting mixed results using other analytical techniques, Cote et al. concluded that the extent of PA detection varied according to the technique employed. Furthermore, their spore-based anti-PA ELISA assays indicated that surface PA could be progressively removed by increasing the extent of purification. The heterogeneous surface PA content measured by FCM and confocal microscopy in our studies are in line with those who attribute its existence to passive contamination. However, unlike the immunoEM study by Cote et al., our assay was not likely to reflect any sub-surface PA since the outermost exosporium functions as a diffusion barrier impermeable to molecules as large as anti-PA (Gerhardt et al. 1972).

**Characterization of *Bacillus* spores by two-color FCM.** We next examined several spore lots to see if differences in preparation, purification, and storage conditions affected the results of the two-color assay (Table 1). The following variables were tested: (1) sporulation media (liquid

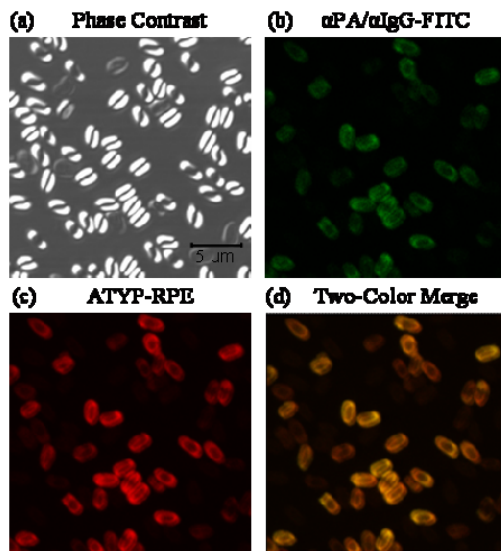


vs. solid); (2) spore purification method (Renografin 76 vs. no Renografin 76); and (3) storage length in ddH<sub>2</sub>O at 4°C (short-term vs. long-term).

<i>Table 1. Characterization of different spore preps by two-color FCM</i>					
<i>Genus / Species / Strain</i>	<i>Lot</i>	<i>D.O.P.<sup>a</sup></i>	<i>%ATYP-RPE</i>	<i>%PA</i>	<i>%DP<sup>b</sup></i>
<i>B. anthracis Sterne<sup>c</sup></i>	BAS7 <sup>1</sup>	05/2007	81	8.0	7.8
<i>B. anthracis Ames<sup>c</sup></i>	BAA1 <sup>1</sup>	02/2007	72	9.7	9.5
<i>B. cereus</i>	BC1 <sup>1</sup>	04/2007	0.070	1.5	0.030
<i>B. thuringiensis</i>	BT1 <sup>1</sup>	04/2007	0.26	0.16	0.080
<i>B. anthracis Sterne</i>	BAS1 <sup>3</sup>	09/2003	12	72	8.6
	BAS2 <sup>3</sup>	09/2003	11	75	6.8
	BAS3 <sup>3</sup>	06/2005	58	36	36
	BAS4 <sup>1</sup>	08/2005	58	33	33
	BAS5 <sup>1</sup>	06/2006	51	23	23
	BAS6 <sup>2</sup>	05/2007	67	32	32
	BAS7 <sup>1</sup>	05/2007	56	32	30
	BAS8 <sup>3</sup>	06/2007	71	43	43
<p><i>a. D.O.P. = date of spore preparation</i>  <i>b. %DP = percentage of double-positive spores</i>  <i>c. assay performed using simultaneous addition method</i>  <i>1. standard liquid sporulation (LD / MGM / Renografin 76)</i>  <i>2. modified liquid sporulation (LD / MGM / no Renografin 76)</i>  <i>3. standard solid sporulation (LD / nutrient agar plate / Renografin 76)</i></p>					

From Table 1 we observed that most *B. anthracis* Sterne spore lots (BAS3-8) showed comparable affinity for ATYP-RPE (51-71% positive) and had similar surface PA values (23-43% positive). We used confocal microscopy to confirm that the double-labeling measured by FCM was not caused by separately labeled spores (Fig. 5). For convenience, detected surface PA was artificially colored green (Fig. 5b), and spore-bound ATYP-RPE was colored red (Fig. 5c). Confocal microscopy is an optical imaging technique which uses point illumination and a spatial pinhole to minimize the detection of out-of-focus light, which allows for increased micrograph contrast and the ability to reconstruct 3-D images. Lots BAS1-2 demonstrated much weaker binding to ATYP-RPE (11-12% positive) and unusually high surface PA levels (72-75%

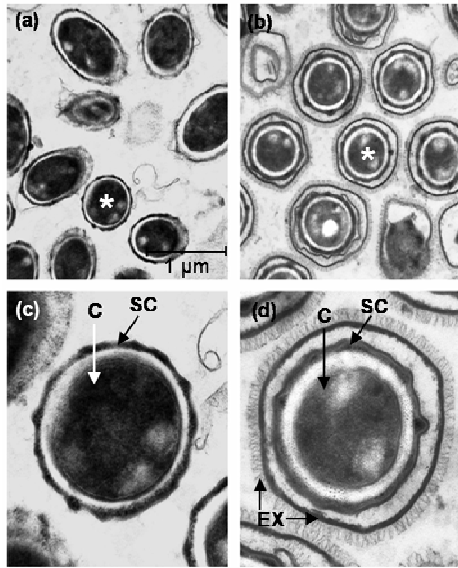
positive). As expected, *B. cereus* and *B. thuringiensis* produced double-negative results by the two-color assay.



**Figure 5.** Phase contrast (a) and fluorescence images of *B. anthracis* Sterne spores treated by the two-color assay. The surface PA fluorescence image (b) and the ATYP-RPE fluorescence image (c) were used to produce the merged image (d).

Neither the sporulation medium nor the purification method affected ATYP peptide binding or surface PA detection. For ATYP peptide binding such a result was expected since neither treatment was considered harsh enough to disrupt the peptide binding site. However, given the proposed existence of surface PA as a passive contaminant, we were surprised to find PA detection was unaffected by the spore lot modifications. These results contradict a previous study which reported the progressive loss of surface PA with increasing extent of purification (Cote et al. 2005). According to that model, one would not expect to find PA on extensively purified spores or on spores which have been stored in ddH<sub>2</sub>O for long periods of time. Our result indicated that surface PA was more securely entrapped in the spore structure than was previously thought, and previous results which form the basis of other models may have simply been a function of the analytical technique employed. Long-term storage (> 4 years) of *B. anthracis* spores in ddH<sub>2</sub>O was found to cause a loss of binding to the ATYP peptide and also a marked increase in detection of surface PA (BAS1-2).

**Investigation of the binding properties of lots BAS1-2 Sterne spores.** Spores from lots BAS1-2 produced unique results by the two-color assay as compared to the other Sterne lots (BAS3-8) (Table 1). Comparison of the two-color FCM data for lots BAS1 and BAS8, which were prepared in exactly the same manner almost four years apart, indicated that physical



**Figure 6.** Transmission electron micrographs of *B. anthracis* Sterne spore lots BAS1 (a and c) and BAS8 (b and d). Asterisks were placed on spores used in the enlarged images. Arrowheads point to the exosporium (EX), spore coat (SC), and core (C). The magnification of images a and b is 21,300x and the magnification of images c and d is 60,000x.

differences may have been responsible for the weak binding to ATYP-RPE and high surface PA content (data not shown) of spores from lot BAS1. Using transmission electron microscopy (TEM), we examined the ultrastructure of spores from lots BAS1 and BAS8 for physical differences that might clarify the binding anomalies seen by the two-color assay (Fig. 6). Freshly prepared lot BAS8 spores contained all the structural

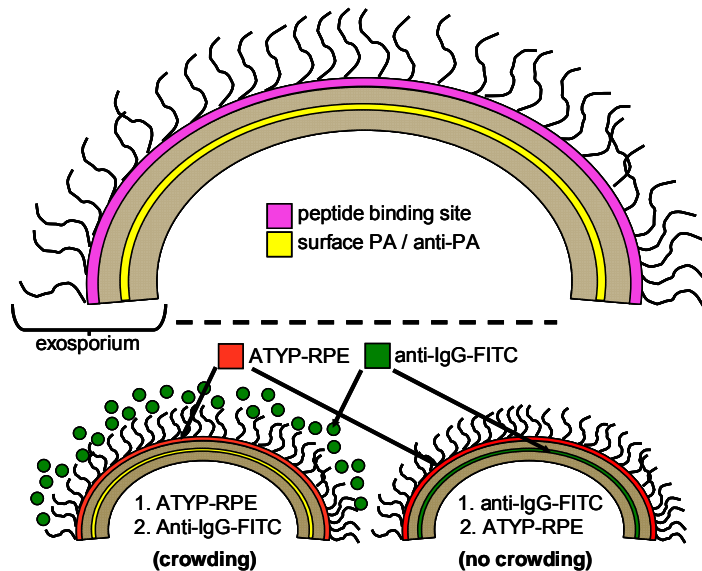
components expected from viable spores, including the core, spore coat(s), and exosporium (Fig. 6b, d). A vast majority of spores from lot BAS1, though

still viable, did not possess an exosporium, and in some instances the spore coat(s) appeared to be degraded as well (Fig. 6a, c).

Although we are unsure what caused the structural damage of lot BAS1 spores, we suspect that either prolonged storage in water or unknown protease activity (possibly through contamination) was responsible. Nonetheless, these ultrastructural images helped define a surface crowding model for binding of ATYP-RPE and anti-PA (Fig. 7). For spores which

lacked an exosporium, ATYP-RPE binding was low and detection of surface PA content was high. This result complimented previous data of ours which indicated that surface PA content was dependent on the order of addition of detection reagents added to anti-PA treated *B. anthracis* spores. Briefly, when ATYP-RPE was added prior to or simultaneous with anti-IgG-FITC, the resulting surface PA values were significantly lower than the average PA value of 39% obtained from single-color studies (Table 1). We hypothesized that the spore binding sites for anti-PA and ATYP-RPE were so close in proximity that binding of ATYP-RPE sterically blocked the binding of anti-IgG-FITC to bound anti-PA. Thus, in order to achieve maximum

detection of surface PA, the anti-IgG-FITC must be added prior to ATYP-RPE.



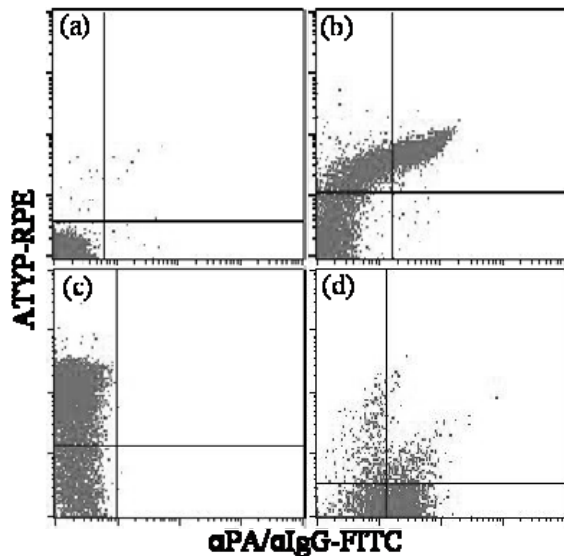
**Figure 7.** Proposed surface crowding model which causes artificially low surface PA values if ATYP-RPE is added prior to or simultaneous with anti-IgG-FITC.

The ATYP peptide binding site was

located on a fragile “sac” layer that covered the spore and may constitute one of four closely packed lamellae that were previously reported to make up the basal layer of the exosporium (Boydston et al. 2006; Gerhardt and Ribi 1964). Given that the exosporium is comprised of a basal layer and an external hair-like nap made exclusively of BclA protein, we concluded that surface PA was also located on the basal layer and its proximity to the peptide binding site caused the observed complications from the two-color assay. Additionally, the absence of the exosporium on lot BAS1 spores meant that anti-PA was free to bind sub-surface PA, which

could explain the increased number of PA-positive spores. This data supports previous claims which suggest PA exists throughout the spore structure and not just in the exosporium (Cote et al. 2005).

**FCM Pattern Recognition.** Finally, based on the results of this study we developed a set of prototypical FCM dot-plot patterns which can be used to predict the species and relative virulence level of unidentified samples of *Bacillus* spores (Fig. 8).



**Figure 8.** Prototypical two-color histogram patterns generated by the study for identifying and characterizing *Bacillus* spores.

Pattern (a) corresponds to non-*anthracis* samples which do not harbor the pXO1 plasmid (i.e. *B. cereus*, *B. thuringiensis*, etc.). Pattern (b) is indicative of spores from those strains of *B. anthracis* which harbor the pXO1 plasmid (i.e. Sterne, Ames, etc.). Pattern (c) is representative of spores belonging to strains of *B. anthracis* which do not harbor the pXO1 plasmid (i.e. Pasteur). Pattern (d) could be generated by one of the following: (1) spores from PA-producing strains of *B. anthracis* which are missing their exosporium; or (2) spores from non-*anthracis* strains which harbor the pXO1 plasmid. The ability to transfer genes across related *Bacillus* species has been achieved in the laboratory, and in some instances, even found to occur naturally. Known as horizontal gene transfer (Helgason et al. 2000), this phenomenon is intriguing from a scientific standpoint, but also one that should be pursued with caution due to its potential role in bioterrorism. We should mention that *B.*

*anthracis* Pasteur was not available for this study, so development of pattern (c) (Fig. 8c) was based solely on work with other strains.

### **Conclusion**

This two-color assay successfully differentiated PA-producing strains of *B. anthracis* from PA-negative strains by FCM in a matter of seconds with minimal sample preparation—a feat that few, if any, spore-based detection strategies can match. However, this technology cannot yet resolve between PA-producing Sterne and Ames strains. Work to identify another spore surface antigen capable of making that distinction is ongoing. Once discovered we envision its use in a three-color FCM assay. Nonetheless, this two-parameter detection system marks an important step toward rapid and complete characterization of the dangerous pathogen known as *B. anthracis*.

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